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Degradation of the polyamine alkaloid aphelandrine by endophytic fungi isolated from *Aphelandra tetragona*

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Abstract

Members of the genus *Aphelandra* (Acanthaceae) produce rare macrocyclic polyamine alkaloids which consist of spermine acylated with two units of 3-(4-hydroxyphenyl)prop-2-enoic acid. Endophytic fungi were isolated from roots and shoots of *Aphelandra tetragona* and tested for their ability to metabolize the main alkaloid aphelandrine, which accumulates exclusively in the roots of the plants. Several endophytes were able to metabolize aphelandrine but only root endophytes belonging to the Nectriaceae were good metabolizers. In addition, the endophytes were grown on an agar medium containing putrescine, spermidine, or spermine as the sole nitrogen source. All fungi were able to grow on putrescine, but only the good aphelandrine metabolizers grew well on spermidine or spermine. *Acremonium* sp. 15, one of the most active metabolizers, grew also on a medium containing aphelandrine as sole nitrogen source. A number of strains thought to be conspecific with *Acremonium* sp. 15 were also tested for their ability to metabolize aphelandrine. The ability of the endophytes to metabolize aphelandrine suggests an ecological adaptation of the symbionts to their host. The possibility of using the aphelandrine metabolism as a taxonomic character is briefly discussed.

Keywords: *Acremonium*; Root alkaloid; Toxin; Chemical adaptation; Chemotaxonomy

1. Introduction

Endophytic fungi have been detected in many vascular plants (for reviews see [1,2]) but little is known about metabolic interactions of the symbiont with the host plant. Grass endophytes are known to produce alkaloids [3] that may protect their host from herbivore grazing and they are known to confer on their hosts resistance to drought and infection by other fungi [4]. Endophytes are also considered to be prime candidates in the discovery of novel metabolites [5].

Polyamine alkaloids are secondary metabolites that have so far been reported from different plant families [6], but their biological function is still unknown. The main alkaloid of *Aphelandra* (Acanthaceae), aphelandrine **1** was first isolated from the roots of *A. squarrosa* and its structure and configuration were determined [7,8]. Biosynthetic studies revealed that the acylated di- and polyamines of type **2** are potential precursors of aphelandrine (Fig. 1) [9]. Phytopathogenic fungi are known to metabolize toxic molecules, e.g. phytoalexins produced by their

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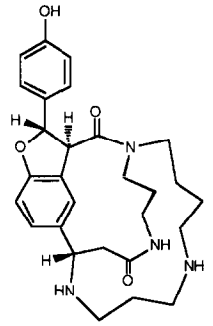
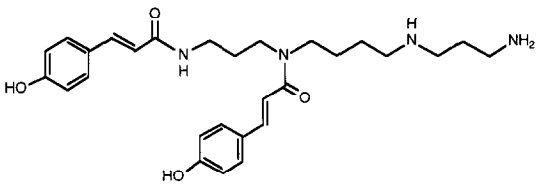
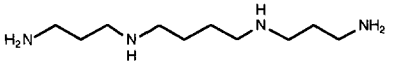


1 Aphelandrine



2 Di-coumaroylspermine



3 Spermine

Fig. 1. Chemical structures of aphelandrine and its precursors.

host plants to less toxic compounds [10,11], but such activity has never been investigated in symptomless endophytic fungi. On the other hand, the adaptation of symbiotic fungi to the constitutive, secondary metabolites of their hosts should be a prerequisite for a successful symbiosis, in particular if such metabolites are fungitoxic.

During our work on the biosynthesis and metabolism of macrocyclic polyamine alkaloids and other *N*-containing secondary metabolites of *Aphelandra* species, we decided to investigate the potential role played by endophytic fungi in the metabolism of these compounds. We isolated endophytes from stems and roots of plants originating from shoot propagation and tested them for their ability to metabolize aphelandrine **1**. Five selected species were tested for their ability to grow on aphelandrine as sole nitrogen source. In addition, we grew all species on an agar medium containing the diamine putrescine or the polyamines spermidine and spermine as sole nitrogen source to obtain some information on the path used by the fungi to metabolize aphelandrine.

2. Materials and methods

2.1. Cultivation of the plants and isolation of the fungi

Plants of *Aphelandra tetragona* (Vahl) Nees were cultivated by shoot propagation in the greenhouse of our institute at the university of Zurich. The plants were grown at 22–24°C, 12 h daylight and a humidity of approx. 80%.

Endophytic fungi were isolated from 40 plants approx. 6–9 months old. From each plant five pieces (5 cm) of the main roots, five pieces (5 cm) of the side roots and two pieces (7 cm) of the lower stem (up to 3 cm above ground) were taken. Surface sterilization of the plant material was performed according to Fisher et al. [12]. Each piece was then cut into five segments that were placed in 90 mm petri dishes containing 2% malt extract agar (Fluka malt extract, 20 g l^{-1}, agar 15 g l^{-1}) supplemented with 50 ppm oxytetracycline hydrochloride (Terramycin®, Pfizer).

Plates were incubated at room temperature for 7–14 days depending on the growth rate of the fungi. Isolation to 2% malt extract agar plates without antibiotic was carried out by transfer of mycelial fragments. After 3 months' incubation most of the isolates sporulated and could be identified.

2.2. Metabolism of aphelandrine

Selected endophytic isolates (Table 1) as well as 15 additional strains received from the Centraalbureau voor Schimmelcultures (CBS; Table 2) and considered to be conspecific with the endophytic isolate *Acremonium* sp. 15 were tested for their ability to metabolize aphelandrine. Two different concentrations of aphelandrine (110 μg and 250 μg per ml agar medium) were used. The agar medium was poured into 24-well plates (1 ml/well) and a mycelial fragment (2×2 mm square) was placed in the middle of each well. Each isolate was tested in three parallels for the two concentrations and the plates were

Table 1
Endophytic fungi isolated from *Aphelandra tetragona* and their ability to grow on polyamines as sole nitrogen source (expressed as a percentage of growth on Czapek medium after 7 days of incubation)

Isolate No.	Taxon	Frequency[a]	Origin[b]	Putrescine	Spermidine	Spermine
1-N[c]	*Fusarium solani* (Mart) Sacc	21	roots	78	90	67
2-O[d]	*Chaetomium* sp. a	17	roots	58	16	0
3-O	*Trichoderma viride* Pers. ex S.F.Gray	16	roots	100	25	15
4-O	*Chaetomium cochliodes* Palliser	15	roots	75	70	20
5-O	*Penicillium pinetorum* Christensen et Backus	12	roots	81	75	62
6-N	*Fusarium* sp.	10	roots	93	77	72
7-N	*Cylindrocarpon destructans* (Zins) Scholten	8	roots	100	71	7
8-N	*Acremonium* sp. 15	7	roots	80	90	30
9-N	*Acremonium* sp. a	7	shoots	80	82	tr.[e]
10-O	*Chaetosphaeria* sp.	7	shoots	97	50	16
11-O	*Humicola grisea* Traaen	6	roots	75	40	0
12-O	*Phialophora* sp.	5	shoots	73	0	0
13-O	*Rhizoctonia* sp. b.	5	roots	89	68	20
14-O	*Acremonium strictum* W. Gams	4	shoots	100	100	47
15-O	*Rhizoctonia* sp. a	3	roots	ND[f]	ND	ND
16-N	*Fusarium oxysporum* Schlecht.	2	roots	90	90	tr.
17-N	*Verticillium* sp.	2	shoots	52	87	tr.
18-O	*Hypoxylon fragiforme* (Pers.: Fr.) Kickx	2	shoots, roots	75	31	0
19-O	*Acremonium* sp. b	1	shoots	80	37	0
20-O	*Gliomastix murorum* (Corda) Hughes	1	roots	71	42	0
21-O	*Calcarisporella* sp.	1	roots	tr.	tr.	tr.
22-O	*Phomopsis* sp.	1	shoots	88	50	13

[a]Total number of plants colonized.
[b]Plant organ from which most isolates were derived.
[c]N: Nectriaceae.
[d]O: Others.
[e]tr.: traces.
[f]ND: not determined.

incubated for 7 days. After this time the surface of the plates was covered with mycelium. The content of each well (agar and fungal culture) was ground in liquid nitrogen, transferred into a centrifuge flask and extracted in 7 ml MeOH:acetic acid (97:3). After 4 h of extraction the suspension was centrifuged and the supernatant collected. The extraction was repeated three times and the extract concentrated in vacuo. The residue was dissolved in 3 ml 1 M glycine buffer pH 9.6, applied to an Extrelut® column (Merck) and eluted with $CHCl_3$. After evaporation of the solvent, the sample was dissolved in a small portion of $CHCl_3$:MeOH (1:1), transferred to an Eppendorf tube (1.5 ml) and dried under N_2. The amount of aphelandrine was determined by HPLC equipped with a UV detector according to Werner et al. [13]. For each experiment three reference samples were extracted without fungal inoculum. The amount recovered from the reference sample was set at 100% recovery.

2.3. Growth experiments on polyamines and aphelandrine as nitrogen source

Selected endophytic fungi were grown on Czapek medium with polyamines or aphelandrine as sole nitrogen source. The amount added was computed to supply the nitrogen amount corresponding to the standard Czapek medium (3 g $NaNO_3$ per liter) [14]. 20 ml medium was poured into plates of 8 cm diameter in the middle of which a small agar block (3×3 mm) with the fungal inoculum was placed. The growth was determined by measuring the diameter of the radial growth after 7 days and expressed as a percentage ratio of the growth on the complete Czapek medium.

Table 2
Origin of strains taxonomically related to *Acremonium* sp. 15 and tested for their ability to degrade aphelandrine, which is shown in Fig. 3

Isolate	CBS Acc. no.	Identity	Origin
A	255.75 B*	*Acremonium* sp.	soil
B	747.83	*Verticillium nigrescens*?	*Apium graveolans*
C	823.73	*Verticillium* aff. *nigrescens*	*Salvinia auriculata*
D	308.38	*Verticillium dahliae* f. *chlamydosporale*	*Apium graveolens*
E	107.75*	*Acremonium* sp.	soil
F	255.75C*	*Acremonium* sp.	soil *Humulus lupinus*
G	706.73*	*Acremonium* sp.	substrate not indicated
H	130.51	*Acremonium apii*	*Apium graveolans*
I	277.89*	*Verticillium* sp.	soil
K	383.66	*Verticillium* aff. *nigrescens*	sugar beet
L	560.65	*Verticillium* aff. *nigrescens*	substrate not indicated
M	413.80*	*Acremonium* sp.	dying fern prothallia
N	711.86	*Acremonium apii*	*Apium graveolens*
O	443.66	*Acremonium restrictum*	moist wall in dark room
P	716.88	*Acremonium restrictum*	human skin
Q	–	*Acremonium* sp. 15	*Aphelandra tetragona*

*Identical with *Acremonium* sp. 15. (W. Gams, personal communication).

3. Results and discussion

We isolated 22 species of endophytes from the shoots and roots of *A. tetragona* (Table 1). The assemblage includes well known endophytes such as *Acremonium* and *Hypoxylon* species as well as members of genera known to contain pathogens such as *Fusarium* and *Rhizoctonia* or saprobes such as *Chaetomium* [15].

The tolerance of endophytic fungi isolated from *A. tetragona* to aphelandrine was tested in a series of experiments. First experiments were conducted by

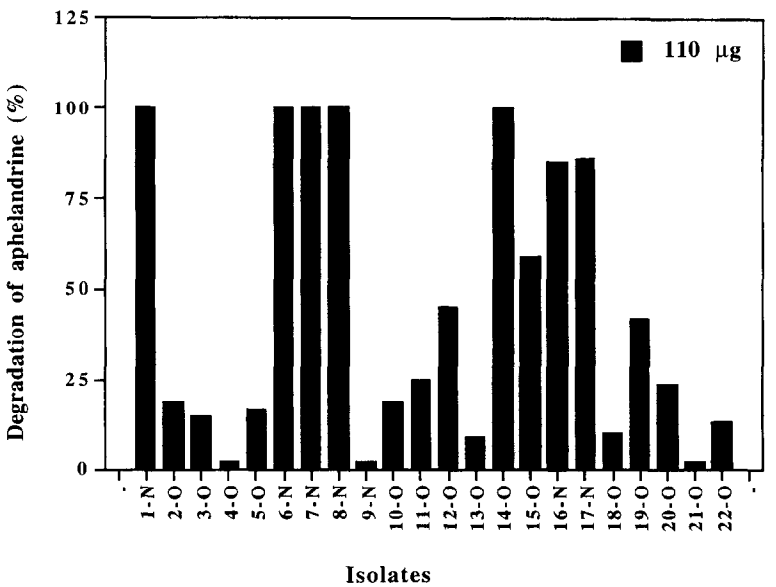


Fig. 2. Degradation of aphelandrine (%) after 7 days of incubation on 110 µg aphelandrine (added to 1 ml malt agar). Identical results were obtained with 250 µg aphelandrine. Endophytic isolates are listed in Table 1.

Table 3
Growth rate (%) of endophytic fungi on the polyamine alkaloid aphelandrine as sole nitrogen source

Isolate	Endophytic fungus	Origin of isolate	Growth (%)[a]
1-N	*Fusarium solani*	roots	60[b]
8-N	*Acremonium* sp. 15	roots	55
9-N	*Acremonium* sp. a	shoots	tr.[c]
14-O	*Acremonium strictum*	shoots	53
19-O	*Acremonium* sp. b	shoots	10

[a]Percentage of growth compared with growth on Czapek medium after 7 days.
[b]The growth of the mycelium is sparse when compared to normal Czapek medium.
[c]tr.: traces.

incubating 32 endophytic isolates on a medium containing a concentration of 10^{-1}–10^{-5} M aphelandrine. The growth of the isolates tested was only marginally affected (data not shown). In a second step, we selected one isolate of each endophyte species, seven of which had been recorded from fewer than three plants (*Acremonium* sp. b, *Calcarisporella* sp., *Gliomastix murorum*, *Fusarium oxysporum*, *Hypoxylon fragiforme*, *Phomopsis* sp. and *Verticillium* sp.) and tested them for their ability to metabolize the polyamine alkaloid aphelandrine (Fig. 2). Of the seven species found to be particularly active, five were mainly isolated from the roots, and four of them were among the most common endophytes of *Aphelandra*. Isolates of *Acremonium strictum* (no. 14-O) and *Verticillium* sp. (no. 17-N) were derived mainly from shoots and only to a lesser extent from roots. A moderate metabolization of aphelandrine was observed for nine isolates, seven of which mainly originated from roots. Only three isolates showed no activity. These results strongly support the hypothesis of an organ-specific adaptation to

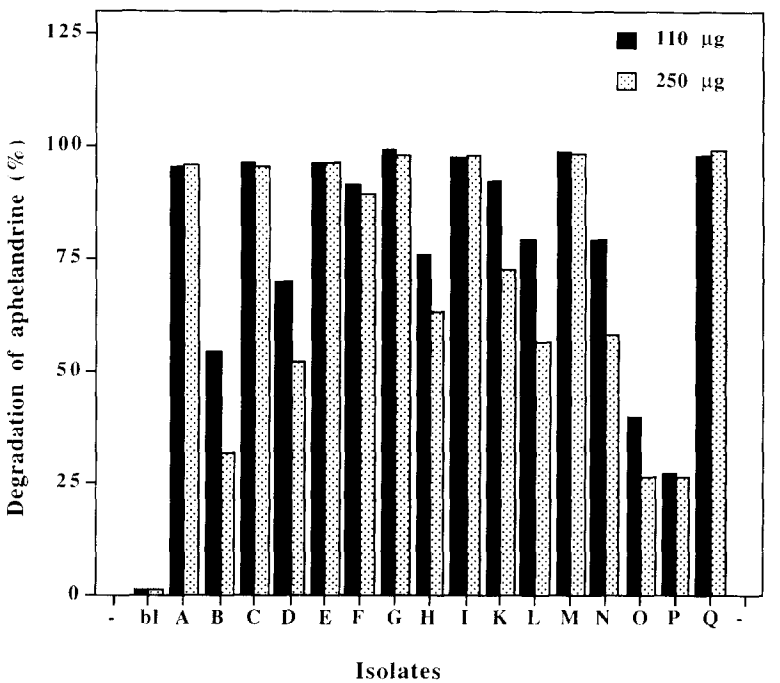


Fig. 3. Degradation of aphelandrine (%) after 7 days of incubation on 110 µg and 250 µg aphelandrine (added to 1 ml malt agar). A–Q: endophytic isolates listed in Table 2. Blank = bl.

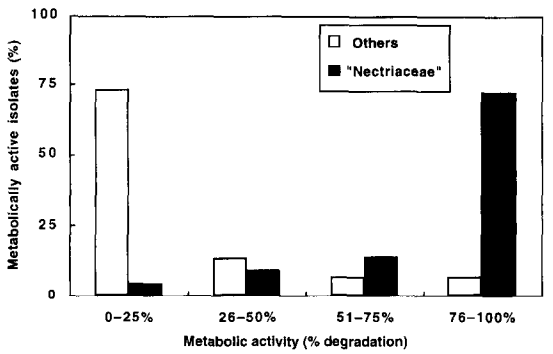


Fig. 4. Metabolic activity (% of aphelandrine degraded after 7 days of incubation) of endophytic fungi according to their taxonomic position.

the microecological and physiological conditions present in a given organ by the *Aphelandra* endophytes, as previously suggested by other studies [16–19].

Fungi can assimilate a wide range of organic and inorganic nitrogen sources. Growth experiments with polyamines as sole nitrogen source were performed with the endophytes to test whether or not a correlation could be seen between the ability of a given isolate to degrade aphelandrine and its growth on polyamines. All fungi were able to grow well on putrescine, irrespective of whether they were active metabolizers or not (Table 1). Growth on spermidine and spermine was apparently species-specific in the set of isolates we studied. Only limited investigations have been performed using polyamines as sole nitrogen or carbon source for fungi [20]. The results of the growth experiments indicate that the endophytic fungi of *Aphelandra* are able to use the polyamine part of aphelandrine as nitrogen source. A selection of endophytic fungi isolated from *A. tetragona* plants was therefore grown on aphelandrine as sole nitrogen source. As aphelandrine is available only in very limited amounts it was not possible to perform a large series of experiments. Therefore, the species chosen were three good metabolizers (isolates 1-N, 8-N and 14-O), a moderately good metabolizer (isolate 19-O) and an inactive isolate (isolate 9-N). The results are shown in Table 3. All good metabolizers were able to grow on aphelandrine. Among them, *Acremonium* sp. 15 (isolate 8-N) was the only one that maintained a normal mycelium and conidiogenesis on the aphelandrine containing medium.

In barley (*Hordeum vulgare* L.) fungitoxic polyamine alkaloids with two units of agmatine connected to two molecules of coumaric acid have been reported [21]. The coumaran skeleton of these molecules is identical to the phenolic part of aphelandrine. When the cells of *A. tetragona* roots are damaged the amount of aphelandrine rapidly decreases [13]. This is due to the activity of a phenoloxidase which in a first step catalyzes a hydroxylation in the *ortho*-position to the existing OH-group on the phenolic part of the molecule. Further oxidation leads to the corresponding quinone followed by polymerization [22]. As poly-phenoloxidases were also reported in fungi [23] and recently detected in *Acremonium* sp. 15 (F. Gabriel, unpublished results), we questioned whether the degradation of aphelandrine by endophytic fungi is due exclusively to phenoloxidation. The ability of the fungi investigated to grow on aphelandrine indicates that the degradation of the polyamine alkaloid is not limited to the phenolic oxidation of the coumaran skeleton but that the polyamine moiety is also metabolized.

The root-inhabiting endophyte species of *A. tetragona* can be divided into two distinct groups. The first includes the highly active metabolizers, all taxonomically belonging to the Nectriaceae, and the second is composed of the moderate metabolizers, not related to the Nectriaceae. With the exception of *Acremonium strictum*, the active metabolizers can all be assigned to the Nectriaceae sensu Rossman [24]. *Cylindrocarpon* spp., *Fusarium* spp., *Verticillium* spp., as well as most *Acremonium* spp. isolated during this study are taxonomically closely related [23]. In fact, *Acremonium* sp. 15 is probably conspecific with some *Verticillium* strains received from CBS (W. Gams, personal communication) and is included in this study for comparison (Table 2). All CBS strains were tested for their capacity to metabolize aphelandrine and nearly all of them were able to degrade the polyamine alkaloid (Fig. 3). Whilst all CBS isolates that are morphologically identical with *Acremonium* sp. 15 (designated Q in Fig. 3) were good metabolizers, the other isolates only partially metabolized aphelandrine (Fig. 3), and the isolates O and P, neither of which originated from plants or soil, were almost inactive. Fig. 4 was prepared by dividing all endophytic isolates into the two groups (O and N, Table 1). All potential anamorphs of

Nectriaceae (N) were the most active metabolizers; all other isolates (O), on the other hand, showed almost no metabolic activity.

Taxonomically closely related fungi are thus apparently able to metabolize the same class of compounds, even if to different extents, thus making utilization of very specialized substrates such as polyamine alkaloids a valuable tool not only in ecology but also in taxonomy. In chemotaxonomic studies, therefore, the ability to utilize special compounds such as aphelandrine could be used to characterize species. In the case of *Acremonium* sp. 15, the metabolism of aphelandrine seems to be a reliable taxonomic character, because all isolates of this species so far investigated are able to degrade aphelandrine.

Acknowledgments

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